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FOLIC ACID

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FOLIC ACID

Six months ago, folic acid was just another member of the somewhat nebulous group of vitamins classified as "newer, less well known factors of the vitamin B. complex." The announcement of its synthesis last August (1) was heralded as a new scientific triumph and speculation as to its significance in human nutrition was heightened with the availability of the pure vitamin for experimental and clinical purposes. There was not long to wait before an answer to such speculation was forthcoming. Quantities of the newly synthesized material were instantly made available for clinical trial on the basis of earlier experimental animal studies and scarcely a month elapsed before a brief note from the Nutrition Clinic in Birmingham, Alabama, appeared in the medical press indicating the dramatic potentialities of folic acid as a potent factor in the therapy of certain types of anemia (2). With each succeeding month, a new chapter was added to the story of folic acid in the management of human anemias, until by January of this year its role as an anti-anemia factor had been firmly established and a preliminary note appeared on its effectiveness in sprue as well, a disease in which anemia is a prominent complication. At the time of this writing, active clinical research is still under way, with new developments expected momentarily. The present status of folic acid indicates it to have striking anti-anemia effects in five types of anemia: nutritional macrocytic anemia*, addisonian pernicious anemia, and the macrocytic anemias of sprue, pellagra and pregnancy (3).

History:

The story of folic acid is less than eight years old. It began in 1938 when Stokstad and Manning (4), who were studying the nutritional requirements of chicks, attempted to devise a diet suitable for biological assay of riboflavin using chicks. In the course of this work they noted that the chicks grew much better on middlings and yeast than could be expected from the "filtrate factor" and riboflavin content of these materials. Almquist, working on vitamin K with chicks at the Division of Poultry Husbandry at the University of California†, had indicated to Stokstad and Manning that he, too, had observed better growth on a diet of natural foods than on a similar diet in which the yeast was replaced by riboflavin, thiamine and filtrate factor concentrates. Obviously, a new, unidentified growth-stimulating factor was present in the crude foods. Detailed studies soon ruled out the possible identity of this factor with any of the known vitamins, including A and D,

*Not to be confused with ordinary "nutritional" or iron-deficiency anemia — *cf. Borden's Review of Nutrition Research,* April, 1944—"Trace Elements in Nutrition. III. Iron."

†*cf. Borden's Review of Nutrition Research,* April, 1943, "The Story of Vitamin K."

vitamin K, and the various B vitamins mentioned above, as well as pantothenic acid and the chick anti-encephalomalacia factor. Lacking sufficient knowledge as to the chemical nature of this new factor it was tentatively designated as Factor U.

The following year in April, 1939, at a meeting of biological chemists, Hogan and Parrott (5) announced the necessity for chicks of what was apparently the same factor — discovered by accidentally substituting an alcoholic extract of liver for the water extract customarily used in their experimental rations. They reported not only subnormal growth as a result of the absence of this new vitamin, but also the occurrence of a severe anemia. The anemia was described as the macrocytic, hyperchromic type (an anemia characterized by abnormally large [*macrocytic*], high hemoglobin, deeply staining, [*hyperchromic*] red blood cells of which there are a markedly decreased number in the circulating blood.*) Unless checked by dietary measures, the anemia resulted in 100 per cent mortality among the animals. Because thee unknown factor apparently belonged to the vitamin B complex, it was designated vitamin B*c*, the subscript *c* indicating its essentiality for the chick (6).

Shortly after these studies with chicks, a new, unidentified factor of the B complex was postulated in the field of bacteriology by Snell and Peterson (7). The factor, found to occur in norite eluates from yeast extracts, was found to be essential for the growth of *Lactobacillus casei* ϵ, *(L. casei)* an organism used for microbiological vitamin assays. Because of its occurrence it was originally termed the "norite eluate factor," but was later designated as the "*L. casei* factor." In 1941, Hutchings and associates (8) made the interesting discovery that the *L. casei* factor promoted growth in chicks. From a comparison of their physical and chemical properties, these investigators suggested that the *L. casei* factor and the previously postulated factor U or vitamin B*c* were, in all probability, one and the same.

With the isolation of the new vitamin in concentrate form by two independent laboratories in 1943 (9, 10), it was possible to demonstrate the mutual identity of factor U, vitamin B*c* and the *L. casei factor.*

In the meantime, soon after the postulation of the *L. casei* factor by Snell and Peterson in 1940 (7), a fourth factor was reported by Mitchell, Snell and Williams. This factor, for which *S. lactis* R was used as the test organism, rather than *L. casei,* was found to have similar properties to the *L. casei* factor. Because a potent concentrate of the new factor was obtained from

*Red blood cells or erythrocytes may vary in total number, in size and in hemoglobin content. These variations may occur in any number of combinations, thus giving rise to a large assortment of characteristic anemias.

spinach and occurred in other leafy materials, it was named "folic acid," from the Latin *folium,* meaning leaf. Folic acid was found to occur in non-leafy sources as well, however, including liver, kidney, yeast and mushrooms. Although folic acid possessed properties closely similar to those of the *L. casei* factor of Snell and Peterson and of Stokstad, it exhibited a number of differences as well. Since none of these materials was available in completely purified form it was difficult to assess the significance of the differences among them, and to determine whether one or more factors were being dealt with. At any rate, the name *folic acid* has come into general, popular usage as a descriptive term for the factor(s) possessing growth stimulating properties for *Lactobacillus casei* and *Streptococcus lactis* R and essential for growth and hemoglobin formation in the chick.*

Related Factors:

The folic acid picture has been considerably complicated by the large number of similar or, possibly, identical factors postulated by various research groups. Widely fluctuating variations in activity have been observed depending chiefly on the source of the vitamin and on the test organism used in its assay.

Microbiological Studies: Thus, there have been factors possessing *L. casei* or "folic acid" activity which exhibited equal potency when assayed with *L. casei* or *S. lactis* R when the test material was derived from liver; a similar material derived from yeast, however, was found to be twice as active for *L. casei* as for *S. lactis* R (10). Similarly, a folic acid concentrate from spinach was found equally active for both micro-organisms, but when derived from other sources, activity was more pronounced, in this case, for *S. lactis* R than for *L. casei* (12). Added to these was still another *L. casei* factor, isolated by Hutchings *et al* (13) from an undisclosed source, found to be almost identical with the liver derivative when assayed with *L. casei* but being only six per cent as active when assayed with *S. lactis* R. In contradistinction to this was a factor described the year before by Keresztesy *et al* (12) as one of their *S. lactis* R-active folic acid concentrates. This factor exhibited such a marked activity for *S. lactis* R — 2500 times as active for this micro-organism as it was for *L. casei* — that it was thought to be an entirely new factor distinct from the other *S. lactis* R-active concentrates.

Hutchings and co-workers (14) assayed a number of different natural food products and observed that some have greater relative potency (as compared with the material isolated from liver) for *L. casei* than for *S. lactis* R; some have the same relative potency, and some have greater relative

*This use of the term "folic acid" is scientifically inaccurate, but persists in the literature because of the convenience of a simple popular name.

potency for *S. lactis* R than for *L. casei:* Some of these have been demonstrated to be identical (e.g. Pfiffner's (9) crystalline vitamin B*c* and Stokstad's (10) crystalline *L. casei* factor from liver); others not yet obtained in crystalline form, may be mixtures of the *L. casei* factor and other unidentified factors with *L. casei* or folic acid activity.

It is interesting to note, however, that a number of these factors upon enzymatic hydrolysis may develop activity for a micro-organism for which they had previously been relatively inactive. Thus, for example, Stokstad's *L. casei* factor (active for *Lactobacillus casei* and for the chick) was found to be extremely potent for *Streptococcus lactis* R following enzymatic treatment (17). Likewise, the highly potent *Streptococcus lactis* R factor of Keresztesy *et al* could be converted into an active *L. casei* factor by enzyme treatment (18). Elucidation of this behavior is still largely in a theoretical state. One explanation, which has already received confirmation in certain instances, may be that the anti-anemia factor is bound up in a large molecule — forming what is known as a conjugate — and is, therefore, unavailable to the organism until released by enzymatic action. This has been shown to be the case in connection with vitamin B*c* from yeast. Yeast concentrates rich in chick anti-anemia activity were found to have very little growth promoting activity for either *L. casei* or *S. lactis* R. Binkley *et al* (19) discovered that digestion of these concentrates with suitable enzymes, however, resulted in the isolation of crystalline vitamin B*c* which is active not only for the chick, but for the two micro-organisms as well. The chick anti-anemia factor in yeast concentrates, accordingly, named vitamin B*c* conjugate (20), has recently been isolated in crystalline form by Pfiffner's group (21) and has been shown to contain vitamin B*c* as an integral part of the molecule.

Animal Studies: Extending the studies of the various folic acid factors to animals has further complicated the picture by introducing still other factors which may or may not be identical with the previously described factors from liver, yeast and spinach. For example, Elvehjem and associates (15, 16) reported a nutritional factor in liver extracts having vitamin B*c* activity (anti-anemia) in chicks, but having little growth activity for the micro-organisms *L. casei* and *S. lactis* R.* The original *L. casei* factor, on the other hand, has been shown to be active not only for *L. casei,* and, on enzymatic hydrolysis, for *S. lactis* R, but, to be a potent anti-anemia factor for the chick (13), the rat (22)† and the monkey (17) as well.

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*In addition, they postulated the existence of two new vitamins in these liver fractions — vitamins B_{10} and B_{11} — essential for proper feathering and growth and probably distinct from the folic acid fraction.

†In the case of the rat, "folic acid" in the form of crude, as well as purified, *L. casei* factor, has cured severe granulocytopenia or anemia, or both, induced by feeding sulfadiazine and sulfathiazole (37, 22). These findings will be discussed more fully in a future Review on the sulfonamides in nutrition research.

Vitamin M:

In the light of these various findings, new consideration was given to an anti-anemia factor for monkeys which had been reported by Day and associates (23) several years before the early studies on the chick anti-anemia factor. This earlier factor, first found in concentrates of yeast and later in liver concentrates, was named vitamin M (24). Because vitamin M had not been isolated or adequately identified, it was impossible to determine its relation, if any, to the *L. casei* factor and it was, at first, not generally considered to be a member of the "folic acid" group.

By 1942, however, a rapidly growing chain of circumstantial evidence was begun which pointed to the close relationship and possible identity of vitamin M with the various microbiological factors of the "folic acid" group. At that time, Wilson and associates in Ohio (25, 26) treated monkeys suffering from nutritional cytopenia (a deficiency of both red and white blood cells) with a "folic acid" concentrate from yeast and obtained a remission of the blood disorder. The following year, Waisman and Elvehjem (27) reported a significant weight increase in monkeys fed a similar concentrate from liver. Without identifying the active fraction, Day's group (28) had, as a matter of fact, shown as early as 1940 that a liver extract would cause remissions in the cytopenic monkey. This group (29) further demonstrated, some years later, that crude vitamin M concentrates when subjected to enzymatic hydrolysis showed good correlation when tested with monkeys and with the micro-organism *S. lactis* R. (N.B. No correlation of activity was evident prior to enzymatic treatment.) With the isolation of an *L. casei* factor in crystalline form by Hutchings *et al* (13), it became possible to apply the crucial test of the identity of vitamin M with the microbiologically active factor: namely, the demonstration that a pure substance, known to be microbiologically active, is similarly active in the monkey.

This experiment was performed and reported three months ago (30). Purified *L. casei* factor, was found to be dramatically effective in the treatment of monkeys made anemic and leucopenic by a vitamin M-deficient diet. This evidence, pending isolation in purified form of vitamin M for final comparative purposes, may be considered tantamount to establishing the identity of vitamin M and the *L. casei* factor. By inference, vitamin M has been closely related also to vitamin B*c* and B*c* conjugate from yeast and to the *S. lactis* R factor (after enzymatic treatment). It has been concluded that for the nutrition of the higher forms of life (chick, rat and monkey) *L. casei* factor, vitamin B*c* and vitamin B*c* conjugate — and, in all probability, vitamin M — are interchangeable.

Additional Related Compounds: Interesting data have accumulated concerning possible metabolic interrelationships between the *L. casei* factor ("folic acid") and certain chemical compounds such as xanthopterin (32,

32), pyracin (33, 34) and thymine (35), to mention a few. The interpretations of these results are still somewhat obscure and controversial, but the observations mentioned here appear to contribute a promising new pathway of research which is now undergoing active investigation.

Occurrence:

"Folic acid," i.e. *L. casei* factor, *S. lactis* R factor, vitamin B*c* and the various related compounds, has been found in a wide variety of natural foods, chiefly liver, yeast, cereal grains (wheat, malt sprouts, oats, ground corn, polished rice, rice bran, linseed meal, alfalfa meal, soybean meal), fish meal, grass juice, spinach, egg white and yolk, bone marrow, meat scraps, milk powder and whey. It is extremely difficult to assign actual "folic acid" values to these various materials because of the wide variations in assay techniques which render interpretation of the findings in terms of *L. casei* factor exceedingly complex. Further, the occurrence of "folic acid" inhibitors, bound forms requiring preliminary enzymatic treatment and compounds capable of partially substituting for "folic acid", are additional factors to be considered in the evaluation of folic acid assays.

Losses of folic acid in cooking have been found to be exceedingly high (36).

Folic Acid in Milk: Studies on the occurrence of inactive or potential folic acid in foods have been reported. These are of particular interest because they constitute an important factor in the difficulties encountered when assaying foods for "folic acid." For example, Wright and co-workers (38) have shown that there exists in milk a form of "folic acid" which is inactive in the microbiological tests (with *L. casei* and *S. lactis* R), but which is active for the rat. They had been previously led to postulate the existence of a microbiologically inactive "folic acid" (39) which possessed vitamin activity for the rat when they found that the incorporation of as much as 10 per cent of one of the vitamin-inhibiting sulfonamides in a ration of powdered whole milk failed to produce the typical folic acid deficiency known to occur when as little as 2 per cent of the drug is added to the usual purified rat diet. This was interpreted as evidence that milk, which on the basis of microbiological tests is quite low in folic acid, contains a component which can be used by the rat in place of the microbiologically active compound. It has been suggested that, theoretically, this might be either a definite substance that the rat organism can use to make its needed folic acid, or perhaps an amino acid or other constituent of milk which favorably influences the intestinal bacteria of the rat to synthesize folic acid. In view of the studies of Binkley *et al* (19, 21) on the occurrence of vitamin B*c* in bound form — vitamin B*c* conjugate — available to the chick but not to *L. casei,* it is possible that a similar compound exists in milk.

Wright *et al* made still further studies on the effects of adding succinylsulfathiazole to a diet of powdered whole milk. They observed that up to 5 per cent of the drug could be added without any adverse effects on growth of the animals over a period of 14 weeks. With 10 per cent of the drug, one rat out of eleven died and the remainder grew slightly more slowly; with 20 per cent, four out of eight rats died within eight weeks and the remainder grew very poorly. Examination of the livers of the animals revealed no evidence of "folic acid" deficiency on the powdered whole milk diet until as much as 20 per cent of the drug had been fed, whereas on a purified synthetic diet decreased "folic acid" levels were noted in the liver when as little as 2 or 5 per cent of the drug was present in the diet. Blood counts at the end of the experiment likewise revealed no abnormalities in animals receiving up to 10 per cent of the drug in powdered whole milk. From these studies, the authors conclude that powdered milk contains significant quantities of "potential folic acid" which is unavailable to microorganisms, but is available to the rat. Although it has not yet been demonstrated, it may be supposed that this form of "folic acid" is available to other higher animals as well, including man.

Synthesis of Folic Acid:

With the isolation of various "folic acid" factors in purified, crystalline form, the way was paved for the elucidation of their chemical structure, and thence to their synthesis in the laboratory. This notable achievement was announced by research workers from Lederle Laboratories and American Cyanamid at a confidential gathering of scientists last July (40) and appeared in print a month later (1). The compound synthesized was announced to be identical with the natural *L. casei* factor isolated from liver. Table 1 and Figure 1 below illustrate the close identity of ultraviolet and infrared absorption spectra for the natural and synthetic materials.

Table 1. Ultraviolet Absorption Spectra of Natural and Synthetic L. Casei Factor

Solvent		mμ	*L. casei* factor from liver $E^{1\%}_{1\ cm}$	Synthetic *L. casei* factor $E^{1\%}_{1\ cm}$
0.1 N NaOH	Minima	235	287	290
	Maxima	256	565	570
	Minima	268	485	495
	Maxima	283	550	560
	Minima	332	133	135
	Maxima	365	195	199
0.1 N HCl	Minima	262	253	265
	Maxima	296	440	445

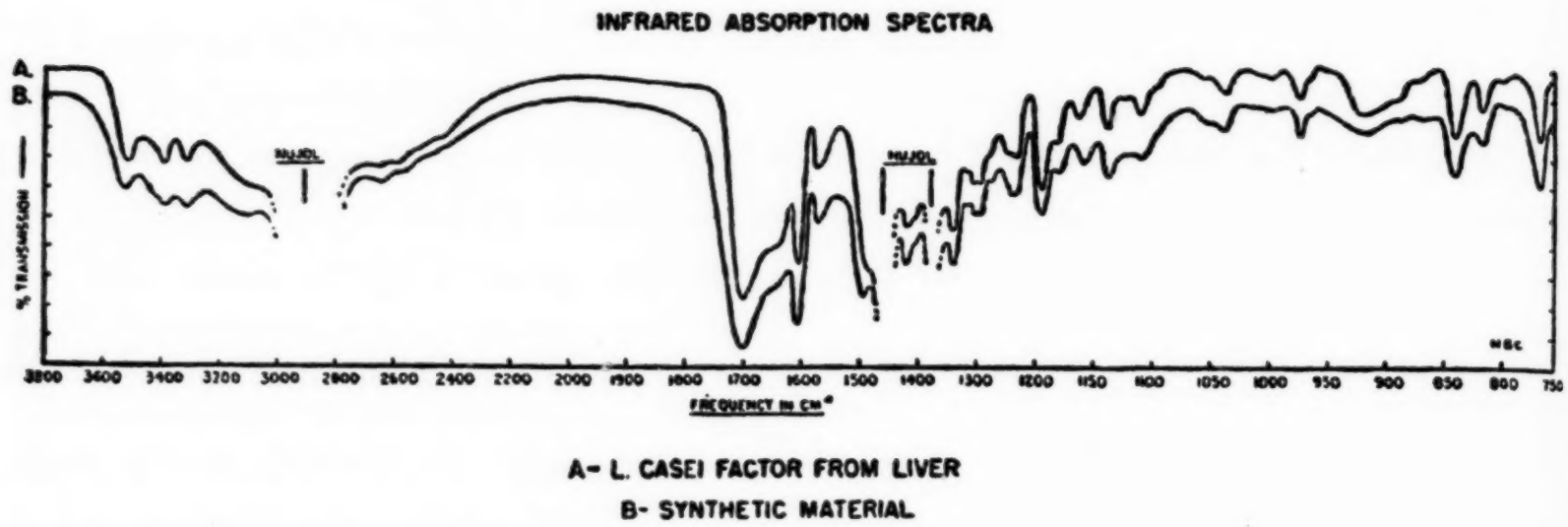

Fig. 1. Infrared absorption spectra of natural and synthetic *L. casei* factors.

In further tests it was shown that the natural and synthetic compounds were equally active when assayed by *L. casei* or *S. lactis* R. Table 2 shows the growth promoting and hemoglobin forming activity of the synthetic compound for chicks as well.

Table 2. Effect of Synthetic L. Casei Factor on Growth and Hemoglobin Formation in the Chick

Supplements per kg ration	Average weight and number alive (–) at 28 days	Average hemoglobin gm per cent. at 28 days
None	93 (5)	4.40
0.5 mg synthetic compound	306 (9)	7.80
1.0 mg synthetic compound	304 (9)	7.36
Fortified stock diet	319 (10)	6.44

Details of the isolation, degradation (to determine structural components) and synthesis are expected to be published shortly, at which time the chemical formula of "folic acid" will be made known.

Immediately following the pre-publication announcement in July of the synthesis of folic acid, limited quantities of the material were made available for clinical research, as well as for further animal studies. By the time synthetic folic acid was announced in the scientific press, preliminary reports were already available on its role in human nutrition as an anti-anemia factor and appeared, almost simultaneously in the medical literature. This demonstrates anew the speedy application of biological research to practical problems in the biochemistry and physiology of man.

In the near future, Borden's Review of Nutrition Research will review the work done with folic acid in the treatment of human anemias.

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NEWS DIGEST

DIETARY DEFORMITIES—The discovery, some eight months ago, that a deficiency of certain amino acids will precipitate a specific deformity of the tongue in chicks has added an additional chapter to the recent story concerning the development of structural abnormalities traceable to faulty nutrition.* During a study of the nutritive value of beef blood proteins it was observed that in chickens fed these materials, the distal or front end of the tongue became folded back onto the dorsal or under surface. This appeared as if the tip of the tongue had been cut squarely off. The cause of the deformity was traced, by microscopic examination, to insufficient development of the median cartilage, resulting in a foreshortened piece which, in turn, caused the folding back of the tongue. A similar deformity was noted in chicks fed diets deficient in the amino acids leucine and phenylalanine. The proteins of beef blood are deficient primarily in the amino acid isoleucine. Control experiments were performed to eliminate inanition and weight loss as causative factors and definitely related the incidence of the deformities to deficiencies of isoleucine, leucine and phenylalanine. These studies have contributed not only to the understanding of the role of diet in developmental abnormalities, but have also contributed to the growing list of specific functions associated with the various amino acids. *Proc. Soc. Exp. Biol. Med. 59,* 177 (1945).

PELLAGRA AND NUTRITIONAL EDEMA — A dietary deficiency disease characterized as an atypical pellagra, and accompanied by nutritional edema, has been reported among non-European children in Johannesburg, Union of South Africa. The disease is characterized by severe liver damage, accompanied by abnormal fatty infiltration; water imbalance; and steatorrhea (disturbance of fat metabolism). Lesions, more or less typical of pellagra, are present (involving mouth, tongue and skin) as well as graying and loss of hair. Strangely enough, the administration of vitamins was entirely without benefit. Liver and raw pancreas occasioned slow and gradual improvement in a few infants over a period of from three to four weeks or more. Astoundingly rapid recoveries were made in all of another group of babies who received small amounts of dried, powdered hog stomach. Edema disappeared within two or three days, followed by rapid healing of the skin lesions. Clinical cures were obtained in ten to fourteen days, including restoration of the fatty livers to normal. The authors suggest the possible therapeutic value of powdered stomach in allied infant diseases such as sprue, celiac disease and nutritional edema and perhaps in other disorders of the liver or of water metabolism. Most remarkable is the fact that normal liver tissue was apparently restored, an occurrence which is not believed possible in infantile pellagra treated with vitamins. *Arch. Int. Med. 76,* 63 (1945).

ELECTRONICS and NUTRITION—Higher nutritive values are now possible in fruits and vegetables processed by electronic heat. Frozen or dehydrated foods require, in the fresh raw state, a preliminary heat treatment to destroy enzymes which would otherwise promote flavor deterioration, loss of color and of vitamins during storage. The commonly used boiling water or steam blanching often damages the texture of the products and leaches out a considereable portion of the water-soluble vitamins. Electronic energy, on the other hand, provides internal heating permitting a vegetable to cook "in its own juice." Using this

*cf. *Borden's Revieew of Nutrition Research,* November, 1944—"Dietary Causes of Congenital Abnormalities."

method, it has been demonstrated, for example, that the usual 30 to 40 per cent loss of ascorbic acid encountered with steam or water blanching of shredded cabbage, can be reduced to a negligible 3 per cent. Further studies promise equally effective processing results for other vegetables and fruits. *Science 102,* 68 (1945).

ANHYDRATED FOODS — Newest members of the processed food family are anhydrated food products. These are partially precooked foods from which the water has been removed by a new, speedier method than heretofore used in ordinary dehydrated foods, thereby resulting in products more nearly like the fresh in flavor, color and aroma. The foods can be stored, without refrigeration, for as long as a year and take up only a fraction of the space which would be required by the same foods in fresh or canned form. A box of anhydrated vegetables about the size of a cigarette carton contains the equivalent of eight No. 2 cans. The anhydrated foods require no preliminary soaking, but are ready for the table within a few minutes after unpacking following cooking with boiling water. Among the new foods processed are mashed potatoes, somewhat like rice in appearance before cooking, and which, after beating up with boiling water for several minutes become fluffy and attractive; orange-colored shreds which cook up in 10 minutes to palatable carrots, and broccoli which cooks similarly. *N. Y. Times,* November 14, 1945.